effective even in absence of conditioning and protected the mice from a disease relevant infection induced by the opportunistic pathogen Pneumocystis murina. Transplantation of functional T cells admixed with HIGM1 T cells resulted in lower vaccination response, indicating competition between WT and HIGM1 cells and implying that increasing the fraction of corrected cells in the graft by selection would improve immune reconstitution. Concerning HSC gene therapy, transplanting 25% WT cells along with HIGM1 ones in HIGM1 mice - mirroring the editing efficiencies achieved in human HSC - rescued antigen specific IgG response and established protection from pathogen comparably to T cell therapy. These findings suggest that autologous edited T cells can provide immediate and substantial benefits to HIGM1 patients and position T cell as competitive strategy to HSC gene therapy, because of more straightforward translation, lower safety challenges and potentially comparable clinical benefits. We thus embarked in assessing GMP compliant reagents and protocols for T cell activation, culture and editing and developed a scalable manufacturing process. Optimization of clinical grade culture conditions allowed further increasing editing efficiency, total cellular yield and maintenance of TSCM thus paving the way to the design of a clinical trial.

39. A Phase 1/2 Study of Lentiviral-Mediated Ex-Vivo Gene Therapy for Pediatric Patients with Severe Leukocyte Adhesion Deficiency-I (LAD-I): Interim Results

Donald B. Kohn[1], Gayatri Rao[2], Elena Almarza[3], Dayna Terrazas[1], Eileen Nicoletti[4], Augustine Fernandes[1],
Caroline Kuo[1], Satiro De Oliviera[1], Theodore Moore[1], Ken Law[4], Brian Beard[4], Julian Sevilla[5], Cristina MesaNunez[6], Claire Booth[7], Adrian Thrasher[7], Juan Bueren[6],
Jonathan Schwartz[4]

[1]UCLA, Los Angeles, CA,[2]Rocket Pharmaceuticals, Inc., Cranbury, NJ,[3]Rocket Pharmaceuticals, Inc, New York, NJ,[4]Rocket Pharmaceuticals, Inc, Cranbury, NJ,[5]Pediatric Oncology Hematology and Stem Cell Transplant Department, FIB Hospital Infantil Universitario Niño Jesús, and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain,[6]Division of Hematopoietic Innovative Therapies, Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación
Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain,[7]Infection, Immunity, & Inflammation Department, UCL Great Ormond Street (GOS) Institute of Child Health, London, United Kingdom

Introduction: LAD-I is a rare disorder of leukocyte (primarily neutrophil) adhesion resulting from *ITGB2* gene mutations encoding for the β2-integrin component, CD18. Severe LAD-I (i.e., CD18 expression on <2% of PMNs) is characterized by severe infections, impaired wound healing, and childhood mortality. Although allogeneic hematopoietic stem cell transplant (alloHSCT) is potentially curative, utilization and efficacy are limited by donor availability and risk of graft-versus-host disease (GVHD). RP-L201-0318 (NCT03812263) is a phase 1/2 open-label clinical trial evaluating the safety and efficacy of RP-L201, consisting of autologous CD34+ cells transduced with a lentiviral vector (LV) carrying the *ITGB2* gene encoding for CD18 (Chim-CD18-WPRE) in severe LAD-I. **Methods:** Pediatric patients

≥ 3 months old with severe LAD-I are eligible. Peripheral blood (PB) HSCs are collected via apheresis after mobilization with granulocytecolony stimulating factor (G-CSF) and Plerixafor and transduced with Chim-CD18-WPRE LV. Myeloablative busulfan conditioning is followed by RP-L201 infusion. Patients are followed for safety and efficacy (i.e., survival to age 2 and at least 1-year post-infusion, increase in PMN CD18 expression to at least 10%, PB vector copy number (VCN), decrease in infections/hospitalizations, and resolution of skin or periodontal abnormalities). **Results:** Four patients (ages 7 months to 9 years) have been treated with RP-L201 with follow-up ranging from 6 weeks to 12 months. RP-L201 cell doses ranged from $2.8x10^6$ to $6.5x10^6$ CD34+ cells/kg with VCNs from 1.83 to 3.8 copies/cell (liquid culture). No serious treatment-emergent adverse events were reported. Neutrophil engraftment was observed in ≤ 5 weeks. PB PMN CD18 expression in Patient 1 12-months post-treatment was 40% (sustained from 47% at 6-months, vs. < 1% at baseline), with PB VCN of 1.2. Skin lesions present at baseline resolved with no new lesions reported. Patient 2 PB PMN CD18 expression 6-months post-treatment was 23% with PB VCN at 0.75. Patients 3 and 4 PB PMN CD18 expression were 74% at 3-months and 59% at 6-weeks post-treatment, respectively. No new infections have been reported in patients post-infusion. **Conclusion:** These results demonstrate that RP-L201 leads to durable neutrophil CD18 expression and improved clinical course. Additional patient treatment is planned for early 2021.

40. Autologous Ex Vivo Lentiviral Gene Therapy for the Treatment of ADA-SCID

Claire Booth[1], Don B. Kohn[2], Kit L. Shaw[2], Jinhua Xu-

[1] SR-TIGET, Ospedale San Raffaele, Milan, Italy,[2]Vita-Salute San Raffaele University, Milan, Italy,[3]Milano-Bicocca University, Monza, Italy

Hematopoietic stem/progenitor cells (HSPCs) gene editing based on homology directed repair (HDR) prospectively allows the treatment of human hematological diseases by *in situ* gene correction while maintaining physiologic regulation. However, in most hematological diseases, gene corrected HSPC would not gain selective advantage over the unedited counterpart; therefore, current HDR editing efficiencies might be insufficient to establish robust therapeutic benefit. Selection of HDR-edited HSPCs, followed or not by ex vivo expansion, would allow administering higher numbers and/or proportions of corrected cells, improving efficacy of HSC therapy while lowering the conditioning requirement and the toxicity of the procedure. Here, we developed selection strategies that couple gene correction and transient Selector expression by Means of Artificial Transcription activators (SMArT). In one design, we fused the

Bayford[3], Elizabeth Garabedian[4], Valentina Trevisan[1], Denise A. Carbonaro-Sarracino[5], Kajal Soni[1], Dayna Terrazas[2], Katie Snell[5], Diego Leon-Rico[1], Karen Buckland[1], Kimberly Gilmour[1], Satiro De Oliveira[2], Christine Rivat[5], Natalia Izotova[1], Stuart Adams[1], Hilory Ricketts[1], Alejandra Davila[2], Chilenwa Uzowuru[1],
Beatriz Campo Fernandez[2], Roger P. Hollis[2], Maritess Coronel[2], Ruixue Zhang[2], Serena Arduini[5], Frances Lynn[5], Mahesh Kudari[5], Andrea Spezzi[5], Marco Zhan[6], Rene Heimke[6], Ivan Labik[6], Kenneth Cornetta[7], Robert Sokolic[4], Michael Hershfield[8], Manfred Schmidt[6], Fabio Candotti[9], Harry L. Malech[10], Adrian J. Thrasher[1], H.
Bobby Gaspar[5]
[1]UCL GOSH Institute of Child Health, London, United Kingdom,[2]University of California, Los Angeles, Los Angeles, CA,[3]Great Ormond Street Hospital NHS Trust, London, United Kingdom,[4]National Human Genome Research Institute, Bethesda, MD,[5]Orchard Therapeutics, London, United Kingdom,[6]GeneWerk, Heidelberg, Germany,[7]Indiana University School of Medicine, Indianapolis, IN,[8]Duke University, Durham, NC,[9]Lausanne University Hospital, Lausanne, Switzerland,[10]National Institute of Allergy and Infectious Diseases, Bethesda, MD

Introduction: Severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) is a rare and life-threatening disorder caused by *ADA* gene mutations leading to compromised immune function. Current treatment guidelines suggest enzyme replacement therapy prior to definitive treatment with hematopoietic stem cell transplantation or gene therapy. An investigational gene therapy (GT) consisting of autologous $CD34^+$ hematopoietic stem cell progenitors (HSPCs) transduced *ex vivo* using a self-inactivating lentiviral vector (LV) encoding the human *ADA* cDNA sequence under the control of a shortened human elongation factor 1α gene promoter (EFS-ADA LV) was studied in trials in the US and EU. Methods: Fifty patients with ADA-SCID (30 in the US and 20 in the EU) were treated with lentiviral gene therapy following nonmyeloablative busulfan conditioning. An analysis was conducted integrating two US studies (using fresh and cryopreserved formulations of OTL-101) at 24-months' follow-up alongside an EU study (fresh formulation) with 36-months' follow-up. Results: Overall survival was 100% for all analyses up to 24 and 36 months. Event-free survival (in the absence of enzyme replacement therapy reinstitution or rescue allogeneic hematopoietic stem cell transplant) was 96.7% (US studies) and 100% (EU patients) at 12 months, 96.7% and 95%, respectively at 24 months, and 95% (EU patients) at 36 months. Engraftment of genetically modified HSPCs persisted in 29/30 US patients and 19/20 EU patients up to last follow-up. Patients exhibited sustained metabolic detoxification and normalized ADA activity levels. Immune reconstitution was robust with T cell counts reaching or approaching normal ranges at last follow up, including increased naïve T cell numbers. 89.7% of US patients and 100% of EU patients discontinuing immunoglobulin replacement therapy by 24 and 36 months, respectively demonstrating improved B cell function. Most adverse events were mild. No evidence of monoclonal expansion, leukoproliferative complications, or emergence of replication-competent lentivirus was noted and no events of autoimmunity or graft-versus-host disease occurred. Conclusions: Treatment of ADA-SCID with *ex vivo* lentiviral HSPC gene therapy resulted in high rates of overall and event-free survival, with sustained *ADA* gene expression, metabolic correction, and functional immune reconstitution.

41. Efficient Ex-Vivo Selection of Gene Edited Human Hematopoietic Stem/Progenitor Cells

Martina Fiumara*[1,2], Samuele Ferrari*[1], Elisabetta Mercuri[1], Aurelien Jacob[1,3], Luisa Albano[1], Angelo Lombardo[1,2], Pietro Genovese#[1], Luigi Naldini#[1,2]

self-cleaving 2A peptide. Since transcription of the edited gene is often low in HSPCs and might not suffice for detectable selector expression, an ArT directed against the targeted gene promoter was co-delivered by mRNA with the editing machinery. Upon editing therapeutically relevant genes in human HSPCs, we achieved transient selector overexpression in all HSPC subpopulations, allowing enrichment of HDR-edited cells up to 90% in the sorted fraction. To avoid selector coexpression in the HSPC progeny physiologically expressing the edited gene, which in some setting may cause toxicity and/or immunogenicity, we improved the SMArT design by introducing in the HDR-template an independent selector cassette regulated by a minimal promoter (minP) that is nearly inactive at basal level. Transient delivery of an ArT binding on the genome flanking the minP but outside the homology region comprised in the HDR-template induced selector expression only in HDR-edited HSPCs. Transactivation, however, was weaker in the more primitive HSPC subpopulations, likely due to the distance between the Art binding site and the transcriptional start site (TSS). To overcome this limitation, we modified the SMArT HDR-template by incorporating multiple tetracycline operator (TetO) repeats close to the minP and transiently co-expressed a tetracyclineregulated TransActivator (tTA) by RNA. Multiplexed ArT binding in close proximity of the TSS allowed robust and transient selector expression in the most primitive HSPC subpopulations. We then tuned tTA activity by an initial pulse of doxycycline administration to suppress transactivation while non-integrated template is still in excess and reached up to 100% HDR editing in the selector-positive fraction. SMArT was portable to several disease relevant loci (*IL2RG*, *AAVS1* and *CD40LG*), compatible with different HSPCs sources and with clinically compliant selectors. On-going experiments are aimed to stringently evaluate the repopulation potential of SMArT-selected HSPCs. Overall, we anticipate that enrichment of HDR-edited HSPCs by SMArT would allow to broaden clinical applicability and tolerability of HSC gene editing.

42. Targeted Genome Editing of Hematopoietic Stem Cells for Treating Recombination Activating Gene 1 (RAG1)

selector (e.g. GFP, ΔNGFR) open-reading frame with the corrective coding sequence of the targeted gene by a

Immunodeficiency

Maria Carmina Castiello[1,2], Nicolò Sacchetti[1], Elena Draghici[1], Samuele Ferrari[1], Valentina Vavassori[1], Chiara Brandas[1], Denise Minuta[1], Martina Di Verniere[1], Aurelien Jacob[1], Enrica Calzoni[1,3], Marita Bosticardo[3], Luigi Daniele Notarangelo[3], Luigi Naldini[1], Anna Villa[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,[2]Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche (CNR), Milano, Italy,[3]Laboratory of Clinical Immunology and Microbiology, NIAID, NIH, Bethesda, MD

Recombination Activating Genes (*RAG*) are tightly regulated molecules during T and B cell differentiation. Mutations in *RAG* genes result in a broad spectrum of immunological disorders including T-B- SCID and leaky forms. Curative treatment is hematopoietic stem cell transplantation but donor availability is still limited and unsatisfactory outcomes have been described with partially HLA-matched donors. Gene replacement therapy have been studied in pre-clinical models, however safety concerns are related to unregulated *RAG1* expression.

Thus, we developed a specific genome editing (GE) strategy, based on the delivery of engineered nucleases and DNA template, to correct *RAG1* mutations and restore gene expression and function. We firstly assessed the minimal therapeutic dose of functional cells by competitive transplant experiments into *Rag1*$^{-/-}$ mice, which showed that low proportion of wild-type or *Rag1*$^{+/-}$ lineage negative bone marrow cells can correct immune defects. We exploited the CRISPR/Cas9 platform combined with the delivery of an AAV6 donor carrying the corrected codon sequence downstream splice acceptor site sequence to use the endogenous *RAG1* promoter. A panel of 12 guides was screened and we selected the best performing one in terms of cutting efficiency assessed by T7 surveyor assay. The selected guide delivered with the corrective AAV6 donor resulted in restoration of RAG1 activity assessed by an in vitro LV-based recombination assay in Rag-1 KO cells. We apply our GE strategy to human CD34+ derived from cord blood or mobilized peripheral blood (MPB) of healthy donors (HD) and we obtained high editing efficiency even in the most primitive hematopoietic stem and progenitor cells (HSPC), assessed as homology directed repair (HDR) efficiency by ddPCR. We exploited an organoid platform to assess the T cell differentiation potential of edited HSPC coupling the GE and the T cell differentiantion protocols optimized for our purposes. Human CD34+ cells edited in *RAG1* locus differentiated in CD3+ TCRαβ+ cells demonstrating that our strategy preserves the T cell differentiation potential. Next, we edited CD34+ cells derived from MPB of HD and two RAG1 patients carrying hypomorpic *RAG1* mutations resulting in a reduced recombination activity and presenting with a combined immunodeficiency with granuloma formation and autoimmunity. Edited cells were transplanted into NSG mice to assess the engraftment capability and kept in culture for phenotypical and molecular analyses. The HPSC phenotype and HDR efficiency (40%) were similar between HD and RAG1-derived edited cells *in vitro.* Importantly, GE did not impact the engraftment and multilineage differentiation of edited HSPC in NSG mice. Moreover, we observed a peripheral selective advantage of edited cells in NSG mice transplanted with edited CD34+ cells derived from RAG1 patients.Overall, our findings suggest that we set up an efficient and promising GE platform for the correction of RAG1 deficiency, that would potentially benefit treatment of RAG1 patients who lack matched donors.

Musculo-skeletal Diseases

43. The Long-Term Efficiency of the scAAV. U7.ACCA Vector in Inducing Dystrophin Expression in Adult Dup2 Mice

Liubov V. Gushchina, Adrienne J. Bradley, Kelly M. Grounds, Aisha Suhaiba, Emma Frair, Calli Bellinger, Tabatha Simmons, Natalie Rohan, Nicolas Wein, Kevin M. Flanigan

Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH

Duplications in exon 2 of the *DMD* gene, encoding the dystrophin protein, account for around 10% of all duplication mutations associated with the X-linked Duchenne muscular dystrophy (DMD). As part of

the preclinical development of a U7snRNA vector currently in a clinical trial, our group has previously evaluated the therapeutic efficacy, absence of off-target splicing effects in AAV9.U7snRNA mediated skipping of exon 2 in a murine Dmd model, and lack of toxicity in non-human primates. Here we report long-term dystrophin expression data following treatment of 3-month-old Dup2 mice with the scAAV. U7.ACCA vector, which contains four copies of U7snRNA targeted to the exon 2 splice acceptor and splice donor sites. Dup2 males received a single intravenous infusion of 3E13 vg/kg, which is the minimal efficacious dose (MED) dose in ongoing first-in-human clinical trial (ClinicalTrials.gov NCT04240314). The age matched Dup2 treated with diluent and C57Bl/6 mice were used as controls. All animals (n=9-11) were sacrificed 18-month post vector administration. The RT-PCR results showed that a single scAAV9.U7.ACCA vector injection resulted in a significant exon 2 skipping in tibialis anterior (TA), diaphragm (Dia) and heart tissues, showing an average of 46%, 32% and 73% total therapeutic transcripts, respectively. To determine the degree of functional rescue, we performed in situ physiology studies on TA and in vitro on Dia muscles. Treated with diluent, both Dia and TA muscles from 21-month-old Dup2 mice exhibited a functional deficit with a significant (45-61%) reduction in specific force output compared with C57Bl/6 (Bl6) mice. The significant force drop was also

observed in diluent treated Dup2 mice compared with Bl6 mice following a rigorous fatigue protocol. The single scAAV9.U7.ACCA injection resulted in a dramatic improvement in specific force output, which increased up to 64-76% in Dia and TA muscles, respectively, and better protection of the TA muscle from repeated fatigue, which improved up to 73%. Overall, these data support our previous findings showing that scAAV9. U7.ACCA provides long-term protection by restoring the disrupted dystrophin reading frame in straight muscles from Dup2 mice and functional recovery of TA and Dia muscles 18-month post vector administration.

44. Towards an Off-the-Shelf Cell Therapy for Bone Healing: Use of an Immortalized, Genetically Modified Cell Line as a Proof of Concept

Rodolfo E. De la Vega[1,2], Michael J. Coenen[1], Gresin Hawse[1], Joseph A. Panos[1], Christopher H. Evans[1]

[1] Rehabilitation Medicine Center, Mayo Clinic, Rochester, MN,[2]cBITE, MERLN Institute - Maastricht Unversity, Maastricht, Netherlands

Introduction: Bone morphogenetic protein-2 (BMP-2) is the most potent clinically available osteogenic cytokine. Yet, after almost two decades in the market, it has limited clinically approved use conditions, requires supraphysiological doses, and side effects are a concern. Gene transfer has been suggested as a means to improve and prolong delivery while lowering side effects. *Ex vivo* gene transfer using autologous cells has been studied in this regard but is limited by the high cost associated with harvesting, tissue culture expansion, transduction, and characterization under GMP conditions prior to re-implantation. This project explores an alternative approach by generating and evaluating a stably-transduced, BMP-2 expressing, immortalized cell line to serve as a proof of concept for an eventual off-the-shelf, allogeneic cell therapy product for bone healing in humans. METHODS: We used HEK293 cells because of their high proliferation rate, ease of maintenance and transduction. Cells were transduced with a lentivirus coding for the human BMP-2 gene driven by the CMV promoter. Clones were selected by puromycin, expanded, BMP-2 production characterized by ELISA and cells frozen at either $5x10^5$ (low), $1x10^6$ (medium) or $3x10^6$ (high) cells/vial. Individual vials were thawed and cells encapsulated in fibrin just prior to surgical implantation in a rat, 5-mm, femoral, critical size bone defect model. The high dose of 11 µg BMP-2 bridges the defect, while empty defects fail to bridge, acting as positive and negative controls, respectively (n=10/group). FK506 was used to prevent xenograft rejection. Defect bridging was monitored via weekly radiographs until euthanasia at 12 weeks. A subset of animals receiving $1x10^6$ cells (n=5) was euthanized at 4 weeks for gDNA extraction from the defect site to determine the presence of implanted cells. RESULTS: Two clones were selected and expanded. Clone CL1K showed significantly higher BMP-2 production per cell than CL20K (Fig. 1A). CL1K was expanded and BMP-2 production was confirmed up to passage 50 (Fig. 1B). After 12 weeks (Fig. 1C), defect bridging seemed to be correlated to the number of implanted cells, with 20%, 60% and 70% of animals bridging in the low, medium and high groups, respectively. qPCR was able to detect gDNA from the implanted cell line in 3 out of 5 animal samples, with one of these animals presenting with solid mass at the femoral defect site. DISCUSSION: Although 293 cells cannot differentiate into osteoblasts, the BMP-2 expressing CL1K cells were able to bridge large bone defects. It is widely assumed that *ex vivo* gene delivery for bone healing must use osteoprogenitor cells as vehicles. However, these data show otherwise and suggest a novel approach to bone healing. We know of no other reports where a cell unable to differentiate into osteoblasts has been used to induce bone healing in a bone defect model. 293 cells are not an ideal candidate for *ex vivo* therapies because of the risk of tumorigenesis, but this experiment serves as a proof of concept for the prospect of an allogeneic off-the-shelf cell line for bone healing. CONCLUSIONS: These novel findings provide a basis for the development of a convenient and effective *ex vivo* gene therapy product for bone healing. Future work is needed to optimize the conditions needed for this approach to be successful for clinical translation.

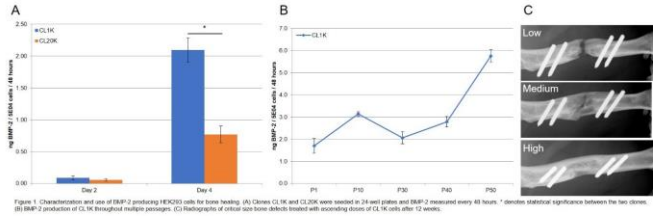

Figure 1. Characterization and use of BMP-2 producing HEK293 cells for bone healing. (A) Clones CL1K and CL20K were seeded in 24-well plates and BMP-2 measured every 48 hours. * denotes statistical significance between the two clones. (B) BMP-2 production of CL1K throughout multiple passages. (C) Radiographs of critical-size bone defects treated with ascending doses of CL1K cells after 12 weeks.

45. Correction of Clcn1 Mis-Splicing Reverses Muscle Fiber Type Transition in Mice with Myotonic Dystrophy

Ningyan Hu, Thurman Wheeler

Massachusetts General Hospital, Boston, MA

Background: DM1 is caused by an expanded CTG repeat in the DMPK gene. Characteristics of this multisystem disorder include myotonia (delayed muscle relaxation), progressive weakness, muscle wasting, and cardiac conduction defects. Clinical features of DM1 arise from expression of DMPK transcripts that contain expanded CUG repeats (CUG)exp that accumulate in nuclear inclusions of skeletal muscle and other affected tissues. This pathogenic RNA readily binds proteins in the muscleblind-like (MBNL) family that are required for normal regulation of alternative splicing, resulting in loss of MBNL protein function. In the HSALR transgenic and Mbnl1 knockout mouse models of DM1, alternative splicing patterns in muscle tissue are very similar. A clinical manifestation of Mbnl1 loss of function includes myotonia, a delayed muscle relaxation caused by mis-splicing of muscle chloride channel Clcn1. In human DM1 muscle, oxidative muscle fibers are upregulated and preferentially atrophic as compared to glycolytic fibers. **Methods**: We crossed HSALR transgenic mice with Mbnl1 knockout mice to create a double homozygous mouse model of DM1. Tibialis anterior muscles were injected with an antisense morpholino oligo that target Clcn1 exon 7a for skipping. The contralateral muscle were injected with the 5' - 3' invert of the active drug. We measured Clcn1 splicing by RT-PCR, transgene expression

by droplet digital PCR, and myosin fiber type by immunolabeling and ddPCR.
Results: In muscles receiving the active treatment, Clcn1 splicing was corrected by 16 days after injection. Type 2B glycolytic muscle fibers were nearly absent in untreated double mutant mice and 50% of the overall total in treated mice. Expression of ACTA1-CUG transcripts and the muscle regeneration marker embryonic myosin (Myh3) was reduced. **Conclusions**: Chronic severe myotonia results in muscle fiber type transition from glycolytic to oxidative in models of DM1. Reversal of Clcn1 mis-splicing is sufficient to rescue muscle fiber type patterns and reduce muscle fiber damage. **Grant support**: Muscular Dystrophy Association.

46. Characterization of Acute Toxicity after High-Dose Systemic Adeno-Associated Virus in Nonhuman Primates, Including Impact of Vector Characteristics

Juliette Hordeaux, Chunjuan Song, Erik Wielechowski, Ali Ramezani, Cecilia Dyer, Elizabeth L. Buza, Jessica Chichester, Peter Bell, James M. Wilson
Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Dose-limiting toxicities have occurred following intravenous administration of high doses of adeno-associated virus (AAV) to target the musculoskeletal or central nervous systems. Acute elevations in liver enzymes and/or reductions in platelets have been observed in most high-dose AAV clinical trials. Although infrequent, severe toxicities have included anemia, renal failure, complement activation, and, in the worst cases, fatal hepatobiliary disease. We previously reported the development of acute thrombocytopenia and transaminitis in most nonhuman primates (NHPs) within days of of receiving high doses of AAV (in the 1x1014 GC/kg range). Some animals recover while some develop a lethal syndrome of coagulopathy, liver failure, hemorrhage, and shock. The acute initial presentation of thrombocytopenia and transaminase elevation is common to NHPs and humans and has not been observed in other species in our experience. Although the progression of the initial acute toxicity may differ in human patients with pre-existing conditions compared to healthy animals, we propose that NHPs can be used as a model to better characterize the acute toxicity of high doses of AAV to reduce risk in clinical trials. We conducted studies aimed at investigating the effect of capsids, prophylactic steroids, and vector purification methods on the

incidence and severity of acute toxicity following high-dose AAV systemic administration. We administered ten rhesus macaques (16 months to 4.6 years old with anti-capsid neutralizing titers < 5 and weighing 3-7 kg) with AAV9 or an engineered capsid derived from AAV9 (AAV-PHP.eB) encoding green fluorescent protein at 1 or 2 x 1014 GC/kg. We then monitored for acute toxicity for 2 weeks post-administration. All vectors were produced from transient transfection of adherent HEK293 cells and either purified using affinity column chromatography or an iodixanol gradient. Both purification methods yielded more than 70% of full capsids. We observed acute toxicity in the majority of animals, regardless of the purification method, characterized by thrombocytopenia, liver enzyme elevation with or without hyperbilirubinemia, and increased coagulation times on day 3 post-administration. Most animals recovered from the initial toxicity except for two AAV-PHP.eB-dosed animals that had to be humanely euthanized due to severe coagulopathy. Prophylactic steroids appeared to help with the recovery from the initial toxicity but did not prevent it from occurring. The toxicity was dose dependent, with AAV9 capsid having a narrow safety margin: 1 x 1014 GC/kg was well tolerated whereas a 2-fold increase led to thrombocytopenia in the majority of animals. Importantly, we observed complement activation concurrent with the thrombocytopenia on day 3 with activation of the alternate pathway and elevation of complement Bb fragment and SC5b-9 membrane attack complex, whereas the classical pathway did not appear to be activated (unchanged C4 and C4a levels). Accordingly, there were no detectable IgM antibodies to the capsid on day 3, suggesting that immune complexes were not the cause of toxicity at 3 days post-administration in NHPs. Collectively, our data suggest that the acute toxicity that occurs after high-dose AAV in NHPs causes thrombocytopenia and liver injury on day 3 post-administration and resolves by day 7 in the majority of animals. This toxicity is capsid dependent, dose dependent, unrelated to a vector purification method, and involves transient activation of the alternate complement pathway.

47. Long-Term Hematopoietic Stem Cell Lentiviral Gene Therapy Corrects Neuromuscular Manifestations in Preclinical Study of Pompe Mice

Niek P. van Til[1,2], Yildirim Dogan[1], Cecilia Barese[1], Zeenath Unnisa[1], Swaroopa Guda[1], Rena Schindler[1], John Yoon[1], Mary Jacobs[1], Abhishek Chiyyeadu[1], Daniella Pizzurro[1], Bianling Liu[1], Mirjam Trame[1], Tim Maiwald[1], Christine Oborski[1], Axel Schambach[3], Claudia Harper[1], Richard Pfeifer[1], Chris Mason[1,4]
[1]AVROBIO, Inc, Cambridge, MA,[2]Child Neurology, Emma Children's Hospital, Amsterdam University Medical Centers, Vrije Universiteit and Amsterdam Neuroscience, Amsterdam, Netherlands,[3]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,[4]Advanced Centre for Biochemical Engineering, University College London, London, United Kingdom

Pompe disease is an inherited disorder caused by acid alpha-glucosidase (GAA) deficiency, leading to lysosomal glycogen accumulation in the heart, skeletal muscles, and the central nervous system (CNS). Pompe disease presents with generalized muscle weakness, and, if untreated, the most severely affected patients typically succumb

early in life due to cardiorespiratory failure. The standard of care is enzyme replacement therapy, which requires lifelong treatment, but does not prevent disease progression. Hence, there is a clear unmet medical need to develop treatment options for Pompe disease patients that could provide a lifelong, life-altering benefit. We used

a lentiviral vector driven approach to overexpress a chimeric human *GAA* transgene with a glycosylation independent lysosomal targeting (GILT) tag in the hematopoietic system of a Pompe disease mouse model. A novel therapeutic lentiviral vector carrying a clinically proven promoter was selected and tested for long-term efficacy in Pompe mice. Supraphysiological GAA enzyme activity levels were achieved in hematopoietic cells and plasma following transplantation with genetically modified hematopoietic stem and progenitor cells (HSPCs) in pre-conditioned Pompe mice. Urinary hexose tetrasaccharides (Hex4), a breakdown product of glycogen, were reduced in treated Pompe mice. GAA enzyme activity was detected in the murine heart, skeletal muscles, spinal cord, and at lower levels in the brain at eight months after transplantation. Glycogen clearance was complete in the murine heart, diaphragm, brain, spinal cord, and in most of the skeletal muscles tested. Consequently, pathological heart remodeling was reversed. Locomotor function was improved. Average vector copy numbers in leukocytes were below five in all the experimental groups. Importantly, administering the lowest dose median VCN ~2 provided robust glycogen reduction indistinguishable from higher doses (median VCN >3) administered. In addition, glucose levels remained stable during the study and the hematopoietic reconstitution of transplanted gene-modified HSPCs was similar to controls. In summary, our candidate vector was shown, in mouse models, to be a potentially effective therapeutic approach for long-term alleviation of cardiomyopathy, muscle weakness and CNS pathology in Pompe disease. We believe this approach could translate into a clinical application of single-dose therapy for Pompe disease patients.

48. Downregulation of the Genetic Modifier *PITPNA* as Means of Therapy in Duchenne Muscular Dystrophy

Matthias R. Lambert, Yuanfan/Tracy Zhang, Janelle M. Spinazzola, Jeffrey J. Widrick, James R. Conner, Louis M. Kunkel

Genetics and Genomics, Boston Children's Hospital, Boston, MA

Duchenne muscular dystrophy (DMD) is a severe genetic disorder caused by mutations in the *DMD* gene. Absence of dystrophin protein leads to progressive degradation of muscle function and leads to premature death. Although there are several promising strategies under investigation to restore dystrophin protein expression, there is currently no cure for DMD, and the development of dystrophinindependent therapies is essential. The recent advances in wholegenome/exome sequencing and the use of large-scale databases have enabled the identification of several genetic modifiers that influence clinical presentation and represent an unexplored territory for therapy. In our lab, we identified *PITPNA* as a new genetic modifier of DMD in two exceptional mildly affected Golden Retriever Muscular Dystrophy (GRMD) dogs. Downregulation of *PITPNA* allowed dystrophindeficient dogs to retain functional muscle and normal lifespan. This was confirmed in our dystrophin-deficient zebrafish in which *pitpna* downregulation by morpholino antisense nucleotide resulted in improved muscle structure and function. In the present study, we explored different strategies that both aimed to downregulate *PITPNA* and prevent the muscle pathology. In dystrophin-deficient zebrafish, we developed a straightforward phenotypic drug screening assay that would be inconceivable in mouse model systems. We identified that phosphodiesterase 10A (PDE10A) inhibitors improved muscle integrity and reduced *pitpna* expression. The PDE10A pathway was confirmed with the use of *pde10a* morpholino and we combined different functional assays that showed improvement in locomotion, muscle, and vascular function as well as long-term survival in *dmd* zebrafish. In dystrophin-deficient mice, we are currently evaluating the potential benefit of *Pitpna* downregulation on muscle pathology based on two different strategies: (i) the use of PDE10A inhibitors and (ii) the use of Pitpna shRNA-AAV. Despite recent advances in dystrophin replacement strategies, there is still precedence to pursue pharmacological therapies targeting genetic modifiers that can complement dystrophin-based therapies and are independent of patients' genetic mutations. In different animal models of DMD, downregulation of the genetic modifier *PITPNA* presents several reasons for there to be further studied of it as a potential DMD therapeutic via two strategies that may benefit patients.

49. Non-Genotoxic Conditioning to Increase Gene Therapy Safety in a Rare Bone Disease

Valentina Capo[1,2], Sara Penna[1,3], Ludovica Santi[1], Andrea Cappelleri[4,5], Stefano Mantero[2,6], Elena Fontana[2,6], Eugenio Scanziani[4,5], Anna Villa[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific

Institute, Milan, Italy,[2]Milan Unit, CNR-IRGB, Milan, Italy,[3]Dimet, University of Milano-Bicocca, Monza, Italy,[4]Department of Veterinary Medicine, University of Milan, Lodi, Italy,[5]Mouse and Animal Pathology Lab (MAPLab), Fondazione UniMi, University of Milan, Milan, Italy,[6]Humanitas Clinical and Research Center

- IRCCS, Rozzano, Italy

Autosomal recessive osteopetrosis (ARO) is a rare genetic disease, affecting osteoclast differentiation or function. Most ARO patients present mutations in *TCIRG1* gene, encoding the a3 subunit of V-ATPase proton pump, necessary for the acidification of bone resorption lacunae by osteoclasts. Symptoms include dense and brittle bones, limited bone marrow cavity, anaemia and progressive nerve compression, leading to death in the first decade of life. To date, hematopoietic stem cell transplantation (HSCT) is the only therapeutic option, but it is limited by availability of HLA-matched donors, toxicity of conditioning regimens and significant morbidity. We and others proposed gene therapy (GT) as an alternative strategy to overcome donor-related issues. However, the burden of conventional myeloablative conditioning on patients remains a strong unmet clinical need. Thus, we evaluated the use of a novel non-genotoxic conditioning in GT setting of *TCIRG1*-dependent ARO. In particular, we tested antibody-drug conjugates (ADCs), formed by anti-cKit or anti-CD45 antibody conjugated to the saporin toxin, able to make space in bone marrow (BM) and to promote hematopoietic stem and progenitor cell (HSPC) engraftment without off-target toxicity in adult mice. Since ARO symptoms occur very early in life, we evaluated the HSPC depleting potential of ADCs on WT newborn mice, administered via temporal vein at 1 day of life. We sacrificed mice 2 days after treatment and observed partial HSPC depletion in BM, spleen and peripheral blood. Notably, we did not

observe histological lesions or increase in apoptosis (evaluated with Caspase 3 immunohistochemistry) due to organ toxicity in kidney, brain and BM of ADC-treated mice. To evaluate the efficacy of non-genotoxic conditioning in favouring HSPC engraftment early in life, we performed mismatched HSCT in WT ADC-conditioned mice, and compared it to transplantation after sublethal total body irradiation or without conditioning. Twenty weeks after transplant, we observed low but persistent donor chimerism in ADC-treated mice compared to irradiated controls in peripheral blood, BM, spleen and thymus.As reported in literature, very low level of donor cells engraftment is sufficient to restore bone phenotype in osteopetrotic mice. We hypothesized that ADC conditioning could successfully guarantee bone phenotype amelioration and reduction of the conditioning toxicity in the osteopetrotic mouse model. This approach may be even more advantageous in the GT setting, in which autologous HSCT avoids the risk of graft rejection. We plan to apply ADC conditioning on osteopetrotic mice before the transplant of lentiviral vector GT Lin- cells, to test the efficacy of our strategy on this severe bone disease model. *Acknowledgements.* This project has received funding from the European Calcified Tissue Society.

Novel AAV Capsids for Brain, Eye and Muscle Tissues

50. Endothelial-Tropic AAVs for Genetic Access to Whole-Brain Vasculature in WildType Mouse Strains Following Non-Invasive Systemic Delivery

Xinhong Chen, Damien A. Wolfe, Sripriya Ravindra Kumar, Timothy F. Miles, Erin E. Sullivan, Acacia M. Hori, Xiaozhe Ding, Viviana Gradinaru

BBE, Caltech, Pasadena, CA

The neurovascular unit (NVU) is a vital yet understudied component of the nervous system. Malfunction of non-neuronal cell types within the NVU, including endothelial cells, can facilitate the progression of neurological disorders (Yu et al, Frontiers in Neuroscience, 2020), but limited options for cell-type specific transgene delivery hamper its study. Adeno-associated virus (AAV) vectors for gene delivery to the brain are commonly administered via intra-cranial injections, resulting in tissue damage and limited, uneven spatial coverage. Systemic AAV delivery provides a non-invasive, brainwide alternative for genetic access. Having engineered vectors that efficiently cross the bloodbrain-barrier (BBB) with broad tropism in rodents (e.g. AAV-PHP. eB), we turned our focus to engineering cell-type-specific vectors that could access vasculature without targeting other components of the NVU. Using M-CREATE directed evolution (Kumar et al, Nature Methods, 2020), we identified a family of endothelial-enriched capsid variants, including one named AAV-CAP.X1. Following intravenous (I.V.) injection, AAV-CAP.X1 targets vasculature with high cell-type specificity and efficiency throughout the body, including the brain. After injecting 3E11 vg total of AAV-CAP.X1 packaging CAG-GFP into adult C57BL/6J mice, 97% (+/- 0.8%) of the GFP+ area in the hippocampus

are CD31+ (demonstrating specificity), and 73% (+/- 9.1%) of the CD31+ area in the hippocampus is GFP+ (proving efficiency; note that an increased dosage of 1E12 vg per mouse resulted in even greater CD31+ labeling without losing specificity). As AAV-CAP.X1 vascular infectivity in the periphery may complicate applications that focus on brain-specific endothelial transduction, we introduced point mutations on the AAV-CAP.X1 capsid and incorporated microRNA target sites into the cargo genome that successfully de-target AAV-CAP.X1 from the liver without impairing brain transduction. AAV-CAP.X1 can be used across multiple genetically diverse mouse strains, with efficient labeling of both capillaries and arteries in the brains of C57BL/6J, FVB/NJ, CBA/J, and BALB/cJ mice following I.V. administration. We also observed a significant increase in transduction compared to its parent capsid AAV9 on multiple human-derived cell lines *in vitro*. In its brain-targeted form, AAV-CAP.X1 could be paired with pre-clinical therapeutic cargo both to probe vascular contributions to neurological disease and to inform intervention strategies. More broadly, gene delivery via endothelial-tropic AAV capsids could, in principle, be applied to study diverse pathologies that may benefit from vascular remodeling. Our evolving knowledge regarding vascular pathology in COVID-19 that could underlie generalized organ dysfunction demonstrates the timeliness and potential importance of such vectors.

51. RNA-Driven Evolution of AAV Capsid Libraries Identifies Variants with High Transduction Efficiency in Non-Human Primate Central Nervous System

Mathieu Nonnenmacher, Shaoyong Li, Wei Wang, Matthew A. Child, Amy Z. Ren, Katherine Tyson, Nilesh Pande, Xiaodong Lu, Jiangyu Li, Xiao-Qin Ren, Jianyu Shang, Michael Hefferan, Jay Hou, Omar Khwaja

Voyager Therapeutics, Cambridge, MA

Widespread transduction of the central nervous system (CNS) by viral or non-viral vectors still represents a considerable challenge in gene therapy. Local delivery of Adeno-Associated Virus (AAV) vectors to the CNS by intraparenchymal or intrathecal administration typically results in strong but heterogeneous transduction and is associated with potential risks related to invasive delivery. By contrast, intravascular delivery of engineered AAV vectors capable of crossing the bloodbrain barrier should allow a broader CNS transduction, thanks to the high density of the brain vascular network. We applied our previously described RNA-driven biopanning TRACER platform to perform directed evolution of an AAV9 capsid library in cynomolgus monkeys (*Macaca fascicularis*). Following two rounds of intravenous dosing and

neuron-specific library selection, a synthetic pool of variants was tested by multiplexed RNA enrichment analysis. This yielded a series of capsid variants with enhanced performance relative to AAV9. Five selected candidates were tested individually by low dose intravenous injection and their tropism for the CNS analyzed by measuring transgene RNA expression, viral DNA biodistribution and immunohistochemistry. All five variants were markedly improved over AAV9, with a subset showing 10-fold or more improvement of transduction in the brain. The highest performing variant displayed more than 1,000-fold higher RNA expression in the brain and 100-fold higher in the spinal cord. Immunohistochemical analysis indicated that this enhanced new

variant displays a predominant neuronal tropism and widespread transduction of multiple brain regions including the cortex, thalamus, putamen and brainstem. Transduction was strikingly high in deep cerebellar nuclei. We propose that this novel capsid variant has potential for use in multiple CNS indications.

52. Expanding the Utility of Intravitreal AAV via a Capsid Variant That Overcomes Neutralization by Anti-AAV2 NAbs in Human Vitreous

Siddhant S. Gupte[1], Sanford L. Boye[1], Wei Li[1], Sergei Zolotukhin[1], Paul Gamlin[2], Siva Iyer[3], Shannon Boye[1]

[1]Pediatrics, University of Florida, Gainesville, FL,[2]Ophthalmology, University of Alabama Birmingham, Birmingham, AL,[3]Ophthalmology, University of Florida, Gainesville, FL

Purpose: Adeno-associated virus (AAV) based gene therapies are approved for treating retinal disorders. While subretinal injection (SRI) leads to efficient gene transfer, intravitreal injection (IVI) is being pursued as a less invasive approach but is complicated by the presence of neutralizing antibodies (NAbs) against AAV in the vitreous. ~70% of the population has pre-existing anti-AAV2 NAbs, which can reduce therapeutic transgene expression to sub-therapeutic levels. As such, the presence of NAbs in the serum is currently used as an exclusion criterion for clinical trials utilizing IVI of AAV. We previously showed that 10% of human vitreous samples screened (31/301) contained high levels of AAV2 NAbs (>95% inhibition of transduction at 1:4 dilution). Here, we evaluated matched vitreous/serum samples (from the same individual) to determine the correlation between respective levels of AAV2 NAbs. We also continued to evaluate the ability of P2-V1, an AAV capsid variant identified through directed evolution and screening in non-human primate (NHP) for the ability to evade neutralization in these samples. Lastly, we characterized the transduction of P2-V1 in macaque following IVI at a clinically relevant dose. The ultimate goal is to identify gene therapy vectors capable of successfully treating the largest proportion of patients. **Approach:** Self-complementary smCBA-mCherry and CBA-GFP vectors were packaged in AAV2, P2V1 or AAV2.7m8 via plasmid transfection. Vectors used in the NHP experiment also underwent affinity column purification. Vitreous and blood (processed to serum) were collected from patients undergoing vitreoretinal surgeries. They were screened for AAV2 NAbs by infecting HEK293 cells with AAV2-mCherry at MOI of $5x10^3$ in the presence of vitreous or serum at 1:4 dilution followed by 4-fold dilutions ranging from 1:10 to 1:10,240. Expression was quantified via flow cytometry. Samples reducing AAV2 transduction >95% at 1:4 dilution were classified as inhibitory. The NAb50 titer (reciprocal dilution at which 50% inhibition of transduction occurs) for each sample was determined. AAV2, P2-V1 and AAV2.7m8 were similarly compared head-to-head in the presence of inhibitory vitreous samples. 1e11vg of AAV2(benchmark) or P2-V1 CBA-GFP vector was IVI into macaque. Transgene expression was recorded in life at 3 and 6 weeks post injection by fluorescent fundoscopy and by immunohistochemistry after sacrifice in retinal sections. **Results:** In head-to-head comparisons using inhibitory vitreous samples, P2-V1 had a 4-64 fold higher NAb50 value than AAV2 in 20 of 29 samples, and AAV2.7m8 in 16 of 22 samples. P2-V1 displayed higher transduction efficiency following IVI in NHP retina compared to AAV2. Of the 19 matched vitreous and serum samples tested so far, 9 serum and 4 vitreous samples inhibited AAV2. All 4 inhibitory vitreous samples had corresponding neutralizing serum, albeit with 4-64 fold higher NAb50. **Conclusions:** P2-V1 outperformed AAV2 and AAV2.7m8 in the presence of human vitreous samples containing anti-AAV2 NAbs and showed higher transduction than AAV2 in NHP retina. While more samples are being assessed, our results with matched serum and vitreous samples suggest that serum NAb levels are a more sensitive measure of a patient's NAb titer than vitreous. We are exploring whether blood/retinal barrier integrity is predictive of higher vitreous NAbs. The ability of P2-V1 to evade neutralization by the matched samples containing anti-AAV2 NAbs will also be studied. Use of P2-V1 may expand the number of patients that can participate in IVI-based clinical trials.

53. Breaking Thru the Human Blood Brain Barrier: Discovering AAV Vectors Targeting the Central Nervous System Using a Transwell Model

Ren Song[1], Katja Pekrun[1], Themasap A. Khan[2], Feijie Zhang[1], Sergiu Pasca[2], Mark A. Kay[1]

[1]Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA,[2]Stanford University School of Medicine, Stanford, CA

Recombinant adeno-associated virus (AAV) vectors for genetic and acquired central nervous system (CNS) disorders continue to gain popularity exemplified by the FDA approval of Zolgensma[R], an AAV9 vector expressing the SMN1 gene from motor neurons for the treatment spinal muscular atrophy. Unfortunately, extremely high doses of the drug are required, leading to varying degrees of adverse effects, including death. A major limitation in the field is the relatively low penetration of vectors across the human blood-brain barrier (BBB). Once across the BBB, selective transduction of various cell types would also be important depending on the type of disorder being treated. In order to enhance human BBB penetration and select for CNS cell selective AAV capsids, we developed two key techniques. First, we used an *in vitro* transwell BBB system with human endothelial cells and separate layers of human iPS cell neurons and astrocytes derived from 3D organoid cultures. We then

tested a pool of 18 known AAV vectors by passaging them through our model system. The pool included AAVs known to cross the BBB more efficiently, such as AAV9 and AAV-rh.10, and those recognized not to cross the BBB (e.g AAV-DJ). The proportion of AAV-9 and AAV-rh10 capsids was enriched in the flowthrough by ~2-fold, while AAV-DJ was reduced by ~5-fold. We created a complex AAV capsid library with ~1 million variants, each containing a unique DNA barcode, using multispecies interbreeding. Three different genetic evolutionary selection schemes were carried out in astrocytes, neurons and the flow thru media. After multiple passages through the humanized BBB transwell system, we used both high-throughput single molecule capsid DNA (2.2kb) sequencing (PacBio) as well as barcode sequencing (Illumina), to identify the capsids that were enriched in the three screens. Multiple enriched AAV variants were made up of complex capsid chimeric sequences that have enhanced ability to pass through the BBB and transduction of astrocytes and/or neurons. We have validated six viral vectors from the screen for astrocytes and all six are able to cross the endothelial cell layer and transduce astrocytes 37 to 91-times better than AAV9. We also found four viral vectors from the screen for neurons that were 3.6 to 20-times better than AAV9 at crossing both the endothelial and astrocyte cell layers. These four vectors are also 1.6 to 4.0-times better than AAV9 at directly transducing iPS-derived neurons. We evaluated the capsids in mice and found several that are liver de-targeted. Our next plan is to pool the best capsids and determine their BBB penetration in non-human primates. Towards this plan, we have selected 6 of the candidates, pooled them together and established their relative BBB penetration in the transwell system, which was predicted as when each was tested individually. Our work supports the use of a human transwell system for selecting improved capsids for CNS based gene therapeutics.

54. Expanding the AAV Toolbox for Cerebellar Transduction: Identifying and Characterizing Novel Variants in Non-Human Primates and Mice

Megan S. Keiser, Yong Hong Chen, Paul T. Ranum, Xueyuan Liu, Congsheng Cheng, David Leib, Geary Smith, Amy Muehlmatt, Luis Tecedor, Beverly L. Davidson

Children's Hospital of Philadelphia, Philadelphia, PA

Introduction: New tools for gene therapy have opened the door to addressing an almost infinite number of genetic lesions, however delivering these tools to the appropriate anatomical location and cell type remains a significant bottleneck. Spinocerebellar ataxias (SCAs) are a family of hereditary movement coordination disorders broadly characterized by neurodegeneration of the cerebellum and brainstem. Here, we characterize the hindbrain tropism of newly identified capsids from an ongoing directed AAV evolution screen in nonhuman primates (NHPs) for future delivery of SCA gene therapies **Methods**: Directed evolution libraries were comprised of AAV1, AAV2, and AAV9 variants generated by addition of random 7-mer peptides into wild-type capsids. Top hits were identified following three consecutive rounds of enrichment based off pooled results from 14 individual brain regions including cerebellar cortex, deep cerebellar nuclei (DCN) and brainstem. Two capsids with enrichment in the cerebellum and brainstem were generated to express fluorescent reporters for visualization of transduction in vivo. Individual variants were delivered to NHPs or adult mice by ICV injection or direct injection in the DCN. **Results**: Following a single ICV injection, a top AAV9 variant was able to transduce Purkinje cells across multiple lobules of the cerebellum as well as a wide variety of other cerebellar cell types including Bergman glia, granule cells, and dispersed interneurons in NHP brain. Injection of the AAV9 variant ICV to adult mice revealed transduction of Purkinje cells. In addition, a top AAV2 capsid variant showed uniformly high levels of Purkinje cell expression in superficial lobules of the cerebellum. Other areas highly transduced in the adult murine brain included the motor cortex, basal ganglia, thalamic nuclei, and brainstem neuronal populations known to be affected in SCA types 1, 2, and 3. Direct parenchymal injections of our AAV2 variant to the DCN of adult mice revealed nearly complete transduction of Purkinje cells in rostral lobules of the cerebellum with positive transduction of multiple brainstem nuclei. Further testing to characterize the biodistribution profiles following different routes of administration

in adult mice and nonhuman primates is ongoing. **Conclusion**: These newly identified variants expand the utilities of AAVs to treat cell types refractory to standard AAV serotypes, and improve targeted delivery following directed or broad administration to treat multiple forms of the spinocerebellar ataxias.

55. Capsid Display of Cell-Penetrating Peptides Yields AAVs with Enhanced Brain Penetration in Both Rodents and Primates

Yizheng Yao[1], Jun Wang[1,2], Yi Liu[1], Yuan Qu[1], Kaikai Wang[1], Yang Zhang[1], Yuxin Chang[1], Zhi Yang[1], Jie Wang[1], Choi-Fong Cho[1], Fengfeng Bei[1]

[1]Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA,[2]Renmin Hospital of Wuhan University, Wuhan, China

Adeno-associated viruses (AAVs) have emerged as promising vectors for gene therapy in the central nervous system (CNS). Engineering of AAV capsid has met with varying degrees of success in generating neurotropic AAV variants, as studies of several AAVs with strong CNS tropism in small model animals fail to translate to large animals such as non-human primates (NHPs). Here, we applied a rational design approach of engineering CNS-penetrating AAVs by displaying cell-penetrating peptides (CPPs) on the AAV capsid. Individual CPPs were inserted into the capsid of AAV9, and the resulting variants were screened for brain transduction after intravenous administration. Peptide sequence optimization by multiple rounds of iteration was performed for leading CPP candidates. Such design approach yielded two variants, namely AAV.CPP.16 and AAV.CPP.21, with 6-249 folds of higher efficiency than AAV9 in brain transduction across four mouse strains. Importantly, the advantage of AAV.CPP.16 over AAV9 for CNS gene delivery translates from mice to NHPs, as AAV.CPP.16

is approximately 5-fold more efficient in systemically transducing the brain in NHPs. In the meanwhile, only a modest increase of efficiency over AAV9 was observed for AAV.CPP.21 in NHPs, in contrast to its more notable brain penetration in mice. Further characterization of AAV.CPP.16 revealed the new variant transduces both neuron and astrocytes in the CNS with notable tropism to the motor neurons in particular. No capsid-associated immuno-toxicity was detected in NPHs based on behavioral monitoring and blood assays, and no cellular abnormality was observed in the dorsal root ganglion cells, which could be associated with potential AAV toxicity. Furthermore, no evidence of break-down of the blood-brain barrier (BBB) by AAV. CPP.16 was observed, and neither AAV.CPP.16 or AAV.CPP.21 binds to Ly6a, a previously identified receptor that is capable of mediating BBB crossing for certain AAV variants. Ongoing studies are further looking into the mechanisms of action on AAV.CPP.16's tropism to CNS tissues. Together, our study reports a novel CNS-tropic AAV vector AAV.CPP.16 with potential applications for gene therapy in both rodents and primates.

Preclinical Gene Therapy for Neurologic Diseases I

56. Engineering AAV6-Based Vectors for Improved Ocular Transduction Following Intravitreal and Intracameral Injection

Sean M. Crosson[1], Shreyasi Choudhury[2], James Peterson[1], Antonette Bennett[3], Diego Fajardo[1], Andras Komaromy[4], Mavis Agbandje-McKenna[3], Sanford L. Boye[5], Shannon E. Boye[1]

[1]Pediatrics, Cell and Molecular Therapy, University of Florida, Gainesville, FL,[2]Ophthalmology, University of Florida, Gainesville, FL,[3]Biochemistry and Molecular Biology, University of Florida, Gainesville, FL,[4]Small Animal Clinical Sciences, University of Michigan State, East Lansing, MI,[5]Pediatrics, Powel Gene Therapy Center, University of Florida, Gainesville, FL

Purpose: The transduction and tropism of AAV in the eye is dependent on vector administration route. Intravitreal injection (IVI) is less surgically complex than a subretinal injection but requires AAVs that traverse the inner limiting membrane, a behavior that relies on the capsid's ability to bind heparan sulfate proteoglycan (HSPG). Transduction of trabecular meshwork (TM) in the anterior segment similarly depends on HSPG binding. The purpose of our study was to rationally engineer AAV6-based vectors for improved transduction of retina and TM following intravitreal or intracameral (ICI) injection, respectively. **Methods:** We evaluated transduction of HEK293, 661W, and rMC-1 cells by AAV1 and AAV6 using flow cytometry. We generated a series of AAV6-based mutants containing previously described proteasomal avoidance (PA) mutations to surface-exposed S, T, and/or Y residues [AAV6(S551V+S663V), AAV6(T492V+S663V), and AAV6(T492V+Y705F+Y731F)] and evaluated their performance in rMC-1 cells. WT mice were IVI with 1E9 vg of AAV6(T492V+Y705F+Y731F), 'aka' AAV6(3pMut), AAV6, or benchmark shH10(Y445F), and evaluated at 6 weeks post-injection (p.i.) by fundoscopy and immunohistochemistry (IHC). Additional variants with increased HSPG affinity or PA, AAV6(3pMut)D532N, AAV6(3pMut)D532R, and AAV6(4pMut) were IVI in mice and evaluated as above. Heparin binding assays were performed using heparin-conjugated agarose beads and gravity flow columns. WT rats received ICI injections of AAV6, AAV6(3pMut), or AAV6(3pMut) D532N (2E9 vg) and the TM, lens, and cornea were imaged by IHC 4 weeks p.i. **Results:** AAV6 variants harboring PA mutations transduced rMC-1 cells with significantly higher efficiency than unmodified AAV6, with AAV6(3pMut) having the highest transduction. In IVI mice, AAV6(3pMut) outperformed both AAV6 and shH10(Y445F) while all had similar tropism for Müller glia (MG) and retinal ganglion cells (RGCs). Addition of D532N and D532R mutations to AAV6(3pMut) increased heparin affinity, more so for AAV6(3pMut) D532R; while the addition of a 4th PA mutation [AAV6(4pMut)] slightly decreased heparin affinity. In IVI mice, AAV6(3pMut)D532N transduction was comparable to AAV6, while AAV6(4pMut) had reduced transduction and AAV6(3pMut)D532R did not transduce. All infectious variants had MG and RGC tropism. AAV6(3pMut)

D532N transduced TM most efficiently, followed by AAV6, then AAV6(3pMut). **Conclusions:** Incorporation of PA mutations onto AAV6 enhances retina transduction via IVI. However, addition of a 4th PA mutation [AAV6(4pMut)] diminished retinal transduction, indicating PA alone is not the sole determinant of transduction via IVI. This reduction is likely attributed to this capsid's decreased HSPG affinity, which may be influenced by the hydrophobic nature of PA mutations. Conversely, retinal transduction by AAV6(3pMut) D532N was comparable to AAV6 but worse than AAV6(3pMut) via IVI, suggesting that increased HSPG affinity counteracts the effects of PA mutations. In TM, AAV6(3pMut)D532N transduction was higher than AAV6 and AAV6(3pMut) suggesting that increased HSPG affinity is a main a driver of TM transduction, rather than PA. Overall, these results suggest that transduction of ocular tissues by AAV requires a balance between the capsid's HSPG affinity and PA.

Preclinical Gene Therapy for Neurologic Diseases I

57. *ST3GAL5* Gene Replacement in CNS Restores Gangliosides Production and Improves Survival in a Mouse Model of GM3 Synthase Deficiency

Huiya Yang[1], Karlla Brigatt[2], Jia Li[1], Kazuhiro Aoki[3], Michael Tiemeyer[3], Robert H. Brown[4], Dan Wang[a5], Kevin A. Strauss[a2], Guangping Gao[a1]

[1]Horae Gene Therapy Center and Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[2]Clinic for Special Children, Strasburg, PA,[3]Complex Carbohydrate Research Center and The Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA,[4]Department of Neurology, University of Massachusetts Medical School, Worcester, MA,[5]Horae Gene Therapy Center and RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA

GM3 synthase (ST3GAL5) deficiency is caused by biallelic mutations of the *ST3GAL5* gene, and results in complete systemic deficiency of GM3 and its downstream a- and b-series cerebral gangliosides (GM1, GD1a, GD1b, and GT1b). The clinical